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Bioaugmentation with Novel Microbial Formula vs. Natural Attenuation of a Long-Term Mixed Contaminated Soil—Treatability Studies in Solid- and Slurry-Phase Microcosms

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Abstract Treatability studies in real contaminated soils are essential to predict the feasibility of microbial consortium augmentation for field-scale bioremediation of contaminated sites. In this study, the biodegradation of a mixture of seven PAHs in a manufactured gas plant (MGP) soil contaminated with 3967 mg kg^{-1} of total PAHs using novel acid-, metal-tolerant, N-fixing, P-solubilizing, and biosurfactant-producing LMW and HMW PAH-degrading bacterial combinations as inoculums was compared in slurry- and solid-phase microcosms over natural attenuation. Bioaugmentation of 5 % of bacterial consortia A and N in slurry- and solid-phase systems enhanced 4.6–5.7 and 9.3–10.7 % of total PAH degradation, respectively, over natural attenuation. Occurrence of 62.7–88 % of PAH biodegradation during natural attenuation in soil and slurry illustrated the accelerated rate of intrinsic metabolic activity of the autochthonous microbial community in the selected MGP soil. Monitoring of the total microbial activity and population of PAH degraders revealed that the observed biodegradation trend in MGP soil resulted from microbial mineralization. In the slurry, higher biodegradation rate constant (k) and lower half-life values ($t_{1/2}$) was observed during bioaugmentation with consortium N, highlighting the use of bioaugmentation in bioslurries/bioreactor to achieve rapid and efficient bioremediation compared to that of a static solid system. In general, natural attenuation was on par with bioaugmentation. Hence, depending on the type of soil, natural attenuation might outweigh bioaugmentation and a careful investigation using laboratory treatability studies are highly recommended before the upscale of a developed bioremediation strategy to field level.

Keywords Manufactured gas plant (MGP) · Soil bioremediation · Polycyclic aromatic hydrocarbons (PAHs) · Heavy metals · Natural attenuation · Bacterial consortium · Bioaugmentation

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1 Introduction

PAHs are ubiquitous environmental contaminants consisting of C and H atoms, arranged in the form of two or more fused aromatic rings (Jacques et al. 2008). Soil contamination with PAHs originated from extensive industrial activities, and these contaminants continue to be generated and released into the environment, particularly through ever-increasing accumulation of residues from oil refineries and gas station containing

complex PAH mixtures including low molecular weight (LMW, two- to three-ringed) and high molecular weight (HMW, three- or more ringed) PAH mixtures (Vandermeer and Daugulis 2007). These compounds are of great environmental concern as they are lipid soluble and quickly absorbed by livings whose metabolism generates products with greater mutagenicity and carcinogenicity (Pereira Netto et al. 2000). Consequently, the US EPA has identified 16 PAHs as priority pollutants and significant efforts are being made to remove PAHs from contaminated lands (Kanaly and Harayama 2010).

Several physical, chemical, and biological methods for decontamination exist, and among those, biodegradation is considered as the best approach to restore PAH-contaminated soils. Bioremediation is a feasible approach for cleaning up PAHs because it is simple, applicable over large areas, cost-effective, eco-friendly, and leads to complete destruction of the contaminant (Bento et al. 2005). Sometimes PAHs remaining in the environment for long periods can be degraded by the autochthonous microbial population in the soil by natural attenuation. When the autochthonous or indigenous microbes do not have the metabolic activity to degrade PAHs, then soil inoculation with exogenous microbes (bioaugmentation) preferably isolated from real contaminated soils that degrade PAHs is a recommended practice (Edgehill 1999). To warrant a practical bioaugmentation application, the primary effect of biodegradation by introduced microbes should deliver a degradation rate greater than the natural rate of decontamination and/or natural attenuation.

One of the practical difficulties in bioremediation using exogenous microbes lies in achieving good or better results in the field as that of the laboratory (Juhasz et al. 2000). Recently, attention has focused on using microbial consortia of PAH-degrading organisms consisting of bacteria because of their enhanced PAH utilization due to metabolic co-operation between organisms involved (Boonchan et al. 2000). However, the soil's physical, chemical, and biological complications can decrease the introduced microbial population mix by antagonistic association (biotic factors), or by predation and opposition from the autochthonous populations, as well as by physiological stresses caused by abiotic factors such as pH, availability of water and air, temperature, and in the most cases for PAHs by the reduced bioavailability of carbon and energy sources (van Veen et al. 1997; Semple et al. 2006; Jacques et al. 2008). Bioremediation efficiency is a function of the capability of the introduced microbial degraders to remain active in the natural environment (Alexander 1999). More recently, the development of consortia with bacterial strains tolerant to several physicochemical conditions such as pH (Wang et al. 2007) and heavy metals (Thavamani et al. 2012), and capable of enhancing PAH bioavailability through biosurfactant production ability (Cameotra and Bollag 2003) is given prime importance so that failure of the introduced microbial mix in soils is curtailed.

In our previous study (unpublished work), we screened individual bacterial cultures with acid and metal tolerance along with biosurfactant production as well as N fixation and P solubilization capabilities. The individual cultures were pooled together, and consortia were developed; the developed consortia exhibited efficient PAH degradation performance in liquid cultures and spiked soil slurries. It is advisable that bioaugmentation of contaminated soils should be preceded by a series of laboratory evaluations, particularly characterization of the microbial populations and the microcosm studies, aiming to evaluate the capacity of selected microorganisms to colonize and degrade the soil pollutant in the real contaminated environment (Sabate et al. 2004; Jacques et al. 2008). Efforts were made in the present study to (a) evaluate the capacity of the developed PAH-degrading consortia to biodegrade PAHs in long-term PAHs and metal mixed contaminated soil (MGP soil) in slurry, as well as solid-phase microcosms; (b) monitor the microbial activity and population during biodegradation; and (c) derive the PAHs biodegradation kinetics in soil.

2 Materials and Methods

2.1 Soil Sample and Microorganisms

A soil sample was collected from the top horizon (1- to 50-cm depth) at a manufactured gas plant site located in Sydney, Australia, which had been contaminated for more than 150 years due to the production of town gas through pyrolysis of coal in retorts. The contaminated soil had the following characteristics: pH 6.8, sand 94 %, silt 3 %, clay 3 %, water-holding capacity 42 %, and contained approximately 3967 mg PAHs kg^{-1} soil. Of the 16 US EPA PAHs, the soil was contaminated with only seven PAHs (mg kg^{-1} soil): acenaphthene

(Ace) 631, phenanthrene (Phe) 449, anthracene (Ant) 117, fluoranthene (Flt) 454, pyrene (Pyr) 2252, benzo[a]anthracene (BaA) 7, and benzo[k]fluoranthene (BkF) 56. Initial characterization also revealed the presence of co-contaminants (heavy metals) in soil (mg kg^{-1}): cadmium 0.4, lead 60, copper 17, and zinc 105. Soil samples were air-dried in the dark, passed through a 2-mm sieve, and stored at ambient temperature (25 °C) until required for use.

The PAH-degrading consortia used for this study are made up of novel acid, metal-tolerant, N-fixing, P-solubilizing, biosurfactant-producing, LMW and HMW PAH-degrading bacteria isolated from MGP soils and koala intestinal gut. Consortium A consists of MGP soil-based strains—*Pseudomonas* (MTS-1), *Stenotrophomonas* (MTS-2), *Agrobacterium* (MTS-4), *Trabulsiella* (MTS-6), and *Cupriavidus* (MTS-7). Consortium N constitutes MGP soil enriched (*Pseudomonas* sp. strain MTS-1 and *Cupriavidus* sp. strain MTS-7) and ample biosurfactant producing koala intestinal gut (*Pseudomonas* sp. strain KC3 and *Bacillus* sp. strain KC5) bacteria.

Some studies (Sabate et al. 2004; Huesemann et al. 2004) indicated that while LMW PAHs (less than four rings) were degraded, five- and six-ring PAHs might also be degraded by co-metabolism with LMW PAHs as substrates, which could stimulate the growth of some specific cells able to degrade five- to six-ring PAHs. Thus, the consortia were maintained by subculturing every 30 days in M9 medium (Thavamani et al. 2015) enriched with Phe and Pyr (100 mg L^{-1} each). About 2-day-old consortia cultured in mild nutrient medium that contained the following (g L^{-1}): glucose 1.25, peptone 1.25, beef extract 0.75 and NaCl 1.25, pH 7 were used as inoculum for microcosm studies.

2.2 Chemicals and Reagents

All chemicals and solvents were purchased from Sigma-Aldrich, Australia. An array of PAH standards were prepared in 100 % methanol. Fluorescein stock and standard solutions were prepared in 60 mM phosphate buffer (pH 7.6). The 60 mM phosphate buffer was prepared in Milli-Q water (18Ω cm^{-1}, Milli-Q, ELGA labwater, UK) and contained the following (g L^{-1}): K_2HPO_4 8.7 and KH_2PO_4 1.3. All stock and standard solutions were stored under refrigeration at 4 °C until use.

2.3 Microcosm Description

To assess the abilities of the bacterial consortia (A and N) to degrade PAHs, experiments were conducted in solid and slurry phases. The treatments were as follows: (1) abiotic control (contaminated soil treated with mercuric chloride, mercuric chloride aids to abate the activity of the microbes present in the soil), (2) unsterile uninoculated contaminated soil (natural attenuation), (3) soil treated with consortium A (bioaugmentation), and (4) soil treated with consortium N (bioaugmentation). Both (1) and (2) served as control.

For PAH degradation in soil, glass jars (2 L) containing 0.5 kg of air-dried soil were inoculated with 5 % of the microbial consortium. The moisture content was adjusted to 50 % water-holding capacity by the addition of MQ water and incubated in dark at room temperature (25 °C). Throughout the incubation period, moisture content of the soil in microcosms was maintained to 50 % water-holding capacity. PAH degradation in slurry was executed alike PAH degradation in soil. The only difference was that the glass jar contained a soil/water ratio of 1:2 *v*/*v* and was retained under shaking (120 rpm). The content of each jar was tilled two times a week for aeration. The microcosm experiments were done in replicate due to the shortage of real contaminated soil. In total, 16 microcosms were set up and incubated for 15 weeks. Periodic sampling for each jar was carried in triplicate at designated time intervals to determine the residual total PAHs and microbial population.

2.4 Soil Analyses

2.4.1 Determination of Total PAHs

Briefly, 5 g of the soil samples were extracted two times in a hexane-acetone mixture (1:1, *v*/*v*) by ultrasonication for 15 min each time in an ultrasonicator operating at 12-kHz sweep bandwith (Soniclean, Australia). PAHs in the combined solvent fractions were concentrated under a stream of nitrogen gas and analyzed for PAHs using an Agilent 1200 Infinity High Performance Liquid Chromatography (DAD/MSD) equipped with a UV detector set at 254 nm. All 16 PAHs were baseline resolved on the Φ 4.6×150 mm reverse-phase C18 column under constant flow rate conditions (1 mL min^{-1}) using a mobile phase gradient consisting of HPLC-grade methanol and Millipore-Q water (80:20, *v*/*v*). Column temperature was maintained at 30 °C.

External calibration was done for individual PAHs using a certified mixture of polynuclear aromatic hydrocarbons (Mix-Ref 4-8095: Supelco, Bellefonte). Reliability of the calibration was checked periodically by injecting known standards and solvent into the column.

Identification and quantification of US EPA PAHs were based on matching their retention time with a mixture of PAH and individual PAH standards. The procedural blank was determined by going through the same concentration. The mean recovery of the PAHs was 90±5.6 %. For the biodegradation experiments, the standard curves were linear in the concentration range of 0.5–20 μg mL^{-1}.

Percentage degradation (D) was calculated using the following formula:

$$D = \frac{PAH_i\text{-}PAH_r}{PAH_r} \times 100 \quad (1)$$

where PAH_i and PAH_r are the initial and residual PAH concentrations, respectively.

2.4.2 FDA Hydrolysis Reaction

Soil microbial activity was estimated by fluorescein diacetate (FDA) hydrolysis. FDA hydrolysis was determined by the amount of fluorescein produced which is directly proportional to the soil microbial population (Adam and Duncan 2001). About 2 g of soil was placed in 50-mL centrifuge tubes, and 15 mL of 60 mM phosphate buffer was added. FDA stock solution (250 μL of 1000 μg FDA mL^{-1}) was added to start the reaction. Blanks were prepared without the addition of FDA substrate along with a suitable number of sample replicates. The tubes were capped, shaken by hand, and were incubated at 25 °C for 90 min.

After 90 min, 15 mL of 100 % methanol was added immediately to terminate the reaction. The tubes were re-capped, and the contents were shaken thoroughly by hand and centrifuged at 3000 rpm for 10 min. The supernatant from each sample was then measured at 490 nm on a Microplate Reader (Synergy™ HT, Bio-Tek). The concentration of the fluorescein released during the assay was calculated using the calibration curve obtained by plotting the concentration vs. absorbance of fluorescein standards (0–5 or 0–50 μg L^{-1}).

2.4.3 Monitoring of PAH-Degrading Microbial Populations

Cell numbers of PAH-degrading bacteria was estimated using the most probable number (MPN) method (Bento et al. 2005). Briefly, 1 g soil was dispensed in 10 mL of MQ water, vortexed thoroughly, and was kept under shaking (120 rpm) for 30 min. Samples were then serially diluted to 10^{-10}. Sterile M9 broth (180 μL) was dispensed into 24-well microtiter plates, and the wells were incubated (five replicates) with 10 μL of the respective dilutions of soil samples. Following inoculation with dilutions of the soil samples, microtiter plates were inoculated with 10 μL of PAH mix. The plates were incubated at 25 °C for 4 weeks. At the end of this period, each plate was scored visually by the development of a brown color (degradation of PAHs) for the PAH degraders. Microbial population was then determined using statistical tables found in Standard Methods of Soil Analysis (Lorch et al. 1995).

2.5 Data Analysis

All the data obtained in this study were subjected to statistical analysis of two-way ANOVA and post hoc Turkey test with SPSS Version 20.

2.6 Bioremediation Kinetics

As kinetics is of great importance because of its prospective to characterize the contaminant concentration remaining at any given time that aids to predict the level likely to be present at some future time, we carried out kinetic analysis for our study. Biodegradability of PAHs is usually expressed by first-order kinetics (Boonchan et al. 2000). and this is given as in Eq. (2):

$$C = C_0 e^{-kt} \quad (2)$$

where C is the residual PAH content in the media at time t (mg L^{-1}), C_0 is the initial PAH content in the media (mg L^{-1}), k is the biodegradation rate constant (day^{-1}), and t is the time (day). Plotting the logarithm of PAH concentration vs. time presents appropriate data about the biodegradation rate.

The biodegradation half-life time ($t_{1/2}$), that is the time taken for a substance to lose half of its amount was calculated by Eq. (3):

$$t_{1/2} = \frac{\mathrm{In2}}{k} \quad (3)$$

where In2 is equal to 0.693 and k is the biodegradation rate constant (day^{-1}).

3 Results

3.1 Degradation of PAHs by Microbial Consortium and/or Autochthonous Microbes

The biodegradation of seven PAHs in MGP soil microcosms lasted for 15 weeks (105 days) in slurry- and solid-phase microcosms, and are shown in Figs. 1 and 2. Apparently, mineralization of significant concentrations of PAHs did not occur in the abiotic control. Indeed, at the end of the experiments, the concentrations in the control soils were measured and found to be ≤10 % of those in natural attenuated and bioaugmented treatments. Briefly, abiotic controls accounted 10 and 6 % of the total PAH degradation in the slurry- and solid-phase systems, respectively. This is similar to the individual and total PAH concentration when the experiment began (zeroth day).

3.1.1 Degradation in Slurry System

Concentration of individual PAHs in the soil slurry under different treatments during the 15-week bioremediation are presented in Fig. 1. All PAH compounds exhibited substantial degradation in both natural attenuated and bioaugmented treatments, which lost 88 and >90 % of total PAHs, respectively. It was notable that significant degradation of most PAHs was observed in natural attenuation. Although significant PAHs degradation was noticed during the phase of natural attenuation, bioaugmentation with consortia A and N enhanced 5.6 and 4.5 % of total PAH degradation, respectively. By the end of the experiment, 6.3 % (consortium N) and 7.4 % (consortium A) of total PAHs remaining was detected in the bioaugmented soils.

In natural attenuation and bioaugmentation, most PAHs showed a steep disappearance during the fifth week of incubation followed by a slow fall in the later phase. No obvious lag periods were observed in the degradation of all PAHs studied—Ace, Phe, Ant, Flt, Pyr, BaA, and BkF. In treatments amended with consortia A and N, the highest PAH removal occurred for Ant (99.5–100 %) followed by Phe (96.2–97.3 %), Ace (95.4–95.6 %), and Pyr (92.9–93.7 %). During natural attenuation, the highest degree of degradation was found in Ace (93.2 %), Pyr (89.1 %), and Phe (87.8 %). In bioaugmentation, removal of Ant, BaA, and BkF was significantly different ($p<0.05$) from that of natural attenuated soils. Over the 15 weeks, the degree of degradation of three-ring PAHs (97 %) was higher than that of four- and five-ring PAHs (86–89 and 83–87 %) in bioaugmented treatments. A similar trend was observed during natural attenuation, i.e., three- (88.1 %) > four- (79.6 %) > five-ring (70 %) PAH degradation.

3.1.2 Solid-Phase Treatments

Results for PAHs concentration vs. time are reported in Fig. 2 for Ace, Phe, Ant, Flt, Pyr, BaA, and BkF in the control, natural attenuated, and bioaugmented solid-phase microcosms. After 15 weeks of incubation, the degradation of total PAHs in treatments incubated with microbial consortium was 72–73.4 %, which was 9–10.7 % higher than natural attenuation (62.7 %). However, like the slurry system, statistical analysis comparing the total degradation for PAHs in the natural attenuation microcosm with rates calculated from the bioaugmented microcosms with consortia A and N indicated that there was no difference in PAH degradation between these three treatments ($p<0.05$). Signficant differences were observed in degradation among the three-ring and between four- and five-ring PAHs ($p<0.05$). The degradation of Ace, Phe, Ant, Flu, Pyr, BaA, and BkF upon incubations with microbial consortia A and N were 88.8–91.1, 62.7–66.5, 54.6–63.1, 77.5–68.5, 68.8–70.9, 69.6–80.4, and 78.2–88.1 %, respectively, with 5.9–8.2, 15.9–19.8, 7.6–16.1, 7.9–16.9, 7.1–9.2, 12.5–23.3, and 24.6–34.6 % increment, respectively, compared to natural attenuation. Maximum degradation of LMW (73.6 %) and HMW (77 %) PAH compounds was observed in bioaugmentation with consortium N. In the solid-phase system, degradation of Ace was rapid in both natural attenuated and bioaugmented microcosms followed by the remaining PAHs compounds in the following order: (a) in bioaugmentation, BkF > BaA > Pyr > Flu > Phe > Ant, and (b) in natural attenuation, Pyr > Flu > BaA > BkF > Phe = Ant.

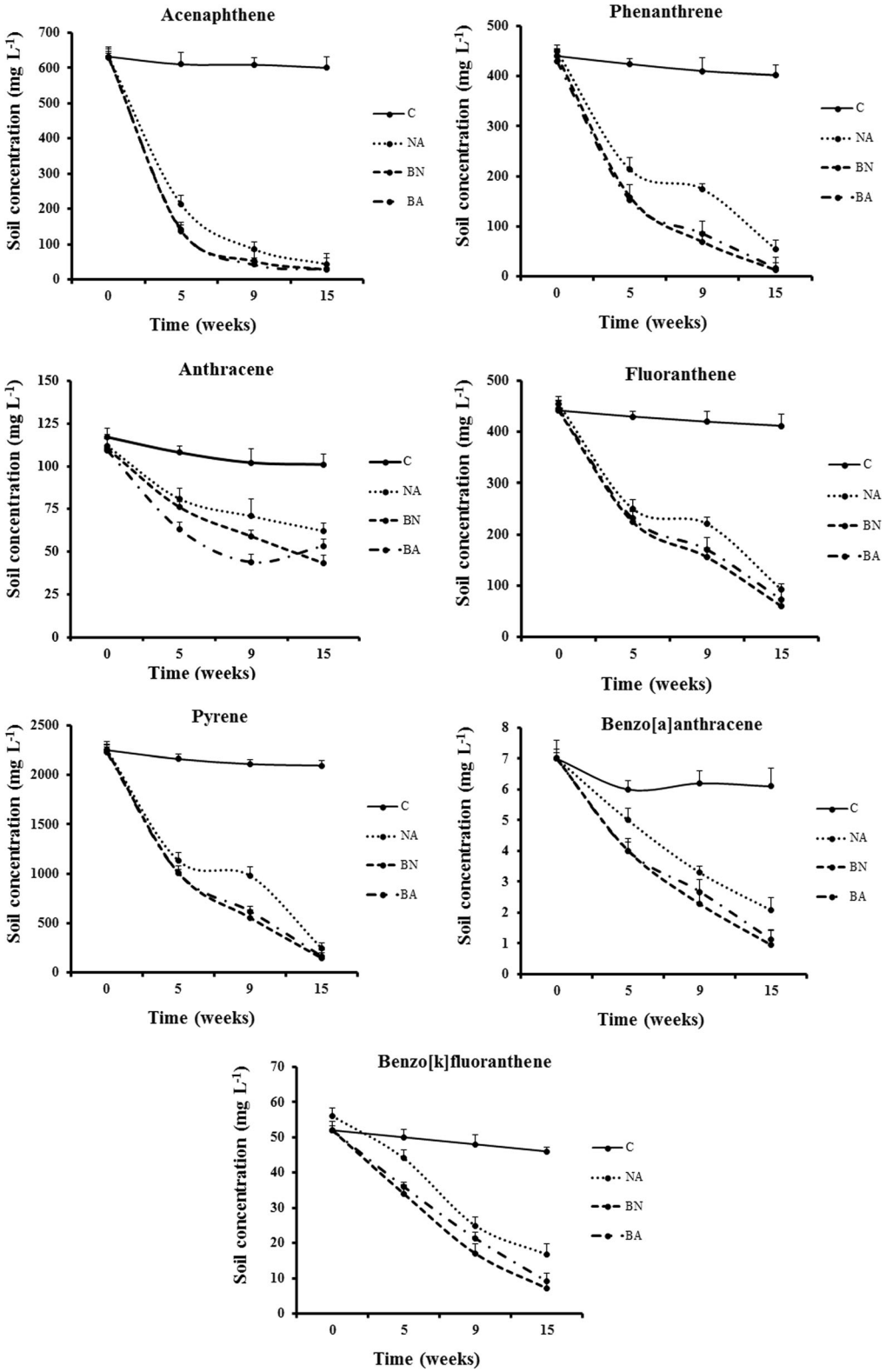

Fig. 1 Biodegradation trend of PAHs in MGP soil slurries enriched with bacterial consortium. *C* abiotic control (soil treated with mercuric chloride), *NA* natural attenuation, *BA* bioaugmentation with consortium A, *BN* bioaugmentation with consortium N

In general, the highest degradation of individual and total PAHs occurred in the slurry phase and not in the solid phase. The increment in the rate and extent of both LMW and HMW PAH biodegradation was 20.6–25.3 % in the slurry system compared to solid-phase microcosms.

3.2 Microbial Growth in Slurry- and Solid-Phase Systems

Microbial biomass is usually used as an index to study the dynamics of pollutant biodegradation. It is determined by the optical density of the liquid culture at a specific wavelength or total cell protein content. However, those methods cannot be employed in the present study because of the use of soil (Wang et al. 2010). So, MPN method was employed to evaluate the total PAH-degrading microbial population in the soil during the bioremediation process in solid- and slurry-phase systems, and the results are shown in Fig. 3a, b. In abiotic control, the size of the PAH degraders seemed to be abated within the second week of incubation with mercuric chloride and remained undetected in the later stages. In contrast with control soils, much higher microbial counts were observed when the soil was naturally attenuated (3.9–4.6 log bacteria g^{-1} soil) and when consortium suspensions (4.4–6.1 log bacteria g^{-1} soil) were added in the solid- and slury-phase microcosms at day 0.

The highest population was detected in slurry-phase microcosms (6.1 log bacteria g^{-1} soil). In both slurry- and solid-phase systems, addition of preselected bacterial consortium (bioaugmentation) resulted in the greatest number (1.3× high) of PAH degraders compared to natural attenuation. Moreover, the fluctuation of the microbial number in the microcosms studied, corresponded highly to the degradation of PAHs. The number of PAH degraders increased quickly accompanied with rapid utilization of PAHs at the initial days, i.e., during the second week when three- and four-ring PAHs were degraded intensively. It continued to increase and reached the peak of 5.7–6.1 log bacteria g^{-1} soil with bioaugmentation and 4.6 log bacteria g^{-1} soil without bioaugmentation in slurry-phase systems in 5 weeks. Then, the number decreased steeply thereafter due to the lack of a carbon source (PAHs).

In solid-phase systems also, the population of PAH degraders showed a fast increase in the first 5 weeks of incubation (natural attenuation=3.9 log bacteria g^{-1} soil, bioaugmentation=4.4–4.7 log bacteria g^{-1} soil) followed by a quick decrease within the next few weeks. Although the bacterial numbers were significantly ($p<0.05$) higher in bioaugmentation than in natural attenuation, the occurrence of substantial degradation in natural attenuation, which was on par with bioaugmentation in slurry- and solid-phase systems, indicates that the removal of pollutants depends more on the quality of the organisms (metabolic activity) than on their quantity.

3.3 Microbial Activity in the Systems Studied

FDA hydrolysis provided a more accurate determination of microbial activity in a wide range of soils (Adam and Duncan 2001). We relied on this test as an index of total microbial population growing in the selected MGP soil. Among the bioremediation treatments in the solid- and slurry-phase microcosms, the highest microbial activity occurred upon bioaugmentation (Fig. 4a, b). The addition of preselected PAH degraders as microbial consortium increased the microbial activity by 1.7–2.1-fold in the beginning (second week) in the slurry-phase system and by 1.2–2.4-fold in solid-phase microcosms. The addition of microbial consortia, stimulated the soil microbial activity until 5 and 7 weeks of incubations in slurry- and solid-phase systems, respectively, and we observed a small decrease in FDA-related activity thereafter. Natural attenuation showed the least microbial activity compared with bioaugmentation.

An approximately nine times higher microbial activity was observed in slurry-phase compared to the solid-phase systems. Also, treatments that received consortium N recorded the highest microbial activity in both systems (slurry phase=68.7 μg g^{-1} fluorescin 3 h^{-1}, solid phase=14.6 μg g^{-1} fluorescin 3 h^{-1}) evaluated. The current trend in microbial activity is analogous to the population of PAH degraders and total PAH degradation. Accordingly, the studies indicate that the observed pollutant degradation in the selected MGP soil is as a result of microbial respiration.

3.4 Evaluation of Biodegradation Kinetics and Half-Life

When residual PAH concentrations were plotted over time, an exponential decay curve was found. So, a first-order reaction model was used to describe the biodegradation behavior of PAHs in MGP soil. The biodegradation rate constant (k) and half-life time ($t_{1/2}$) values of

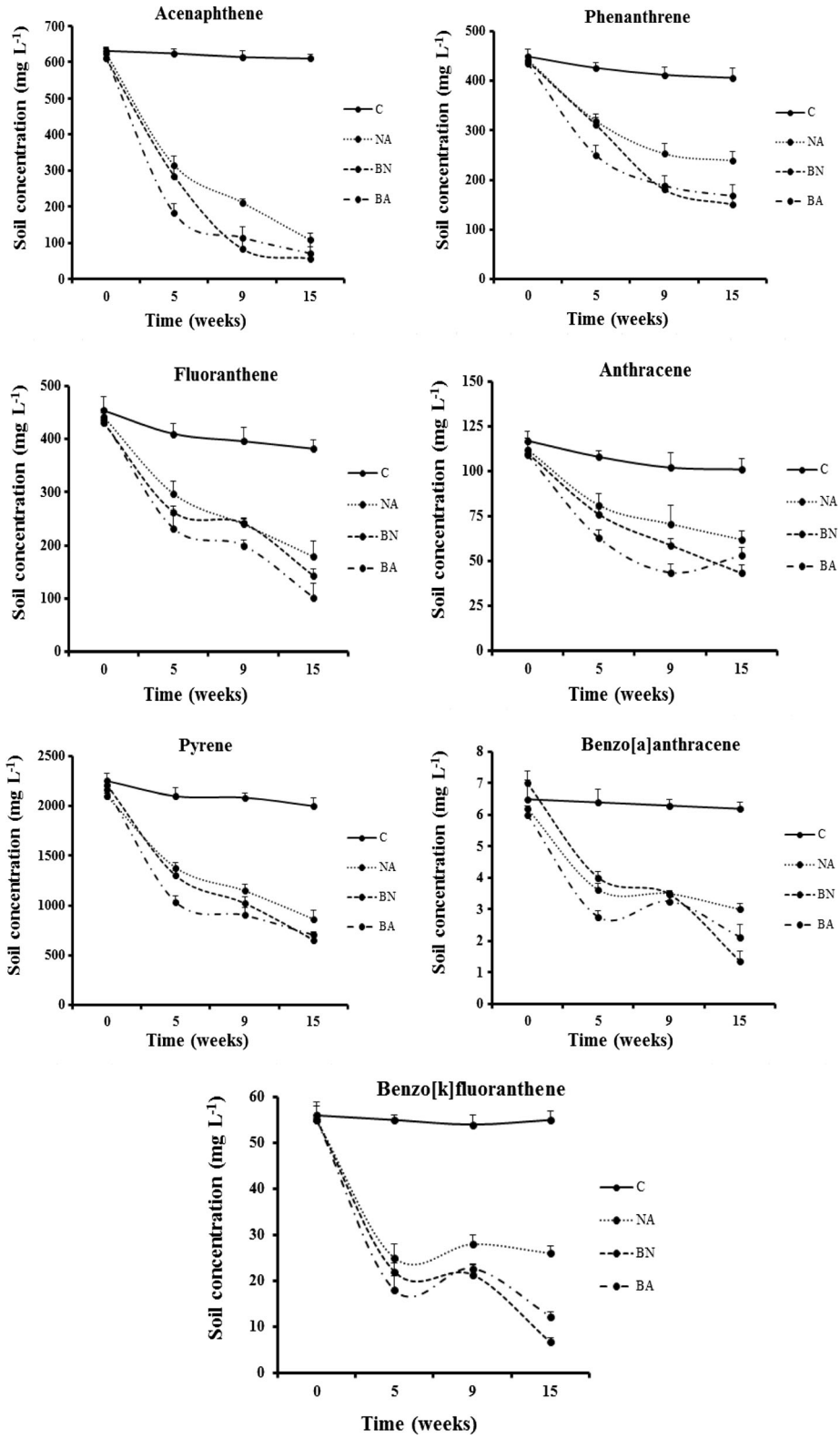
Acenaphthene
Phenanthrene
Fluoranthene
Anthracene
Pyrene
Benzo[a]anthracene
Benzo[k]fluoranthene
Soil concentration (mg L-1)
Time (weeks)
C
NA
BN
BA

Fig. 2 PAH biodegradation trend of a long-term mixed contaminated soil enriched with bacterial consortium and/or autochthonous microbes in solid-phase microcosms. *C* abiotic control (soil treated with mercuric chloride), *NA* natural attenuation, *BA* bioaugmentation with consortium A, *BN* bioaugmentation with consortium N

PAHs in solid- and slurry-phase microcosms are shown in Table 1. The larger value of R^2 suggested a better fit for both systems evaluated. The half-lives of PAHs ranged from 8 days for Ace to 19 days for BkF in slurry-phase microcosms and from 10 days for Ace to 41 days for Ant in the treatments of solid-phase microcosms. The biodegradation rate constants in the treatments of slurry-phase microcosms were generally larger, and half-lives were almost smaller (up to 3.3-fold) than those of solid-phase systems, indicating that the biodegradation of PAHs in the slurry was faster than in solid phase, as previously mentioned.

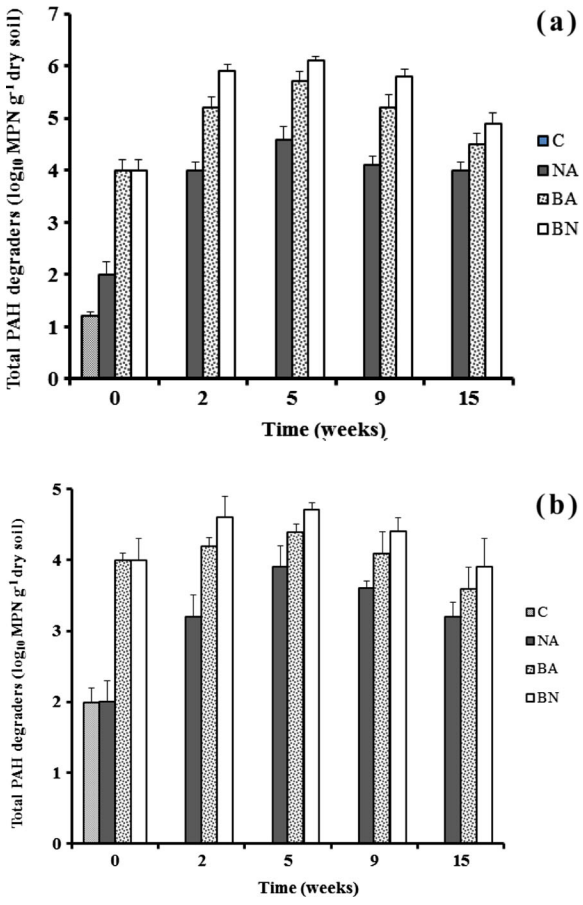

Fig. 3 Changes in population size of PAH degraders in soil microcosm that did and did not receive microbial consortium. **a** Slurry-phase microcosm. **b** Solid-phase microcosm

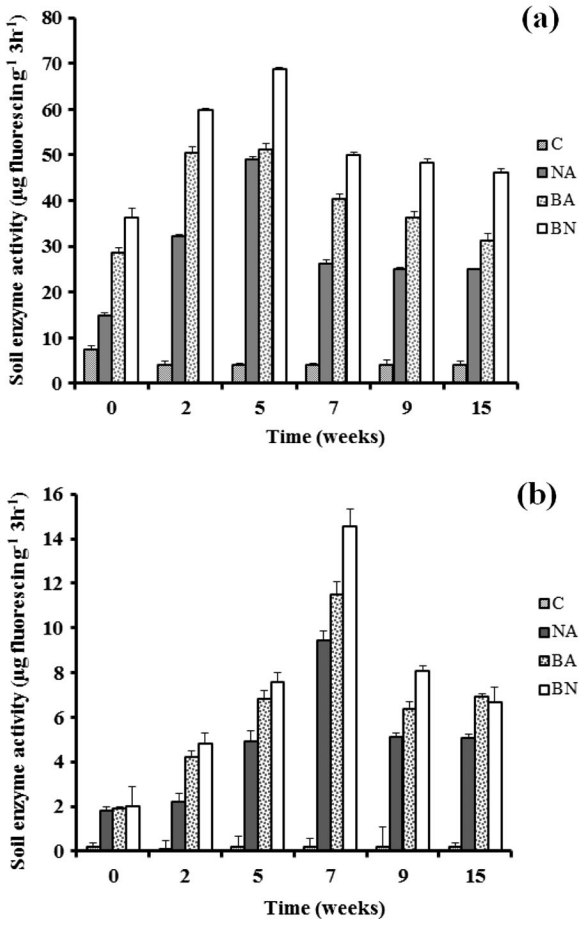

Fig. 4 Microbial activity in soil microcosms unamended and amended with bacterial consortium. **a** Slurry-phase microcosm. **b** Solid-phase microcosm

The expected increase in half-life time with increasing molecular weight of PAHs was not observed, particularly during biodegradation in solid-phase system. This may be related to the bioavailability as well as the total concentration of PAHs in the soil. The half-life values of HMW PAHs were 1.3 to 3× higher than LMW PAHs in most cases, showing that HMW PAHs are more persistent in soils than LMW PAHs with three aromatic rings. Significant ($p<0.05$) differences were not observed in the half-lives of individual PAHs in the slurry-phase treatments. Significant difference ($p<0.05$) in the half-lives of Phe, Ant, BaA, and BkF in the solid-phase treatments that were natural attenuated and bioaugmented were observed. In solid-phase microcosms, the degradation of Phe, Ant, BaA, and BkF were 1.2–1.6, 1.2–1.3, 1.4–1.7, and 1.5–2.1 times higher during bioaugmentation with consortia A and N than natural attenuation. In both solid- and slurry-phase

Table 1 The biodegradation rate constants (k) and half-life ($t_{1/2}$) time of PAHs in solid- and slurry-phase microcosms

Kinetic parameter	Acenaphthene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo[a]anthracene	Benzo[k]fluoranthene
Slurry-phase system							
(a) Natural attenuation							
k (day^{-1})	0.079	0.059	0.052	0.044	0.061	0.036	0.037
R^2	0.98	0.97	0.99	0.96	0.93	0.99	0.97
$t_{1/2}$ (days)	9	12	13	16	11	20	19
(b) Bioaugmentation with consortium-A							
k (day^{-1})	0.091	0.093	0.154	0.051	0.075	0.053	0.052
R^2	0.91	0.99	0.93	0.99	0.98	0.99	0.98
$t_{1/2}$ (days)	8	7	4	14	9	13	13
(c) Bioaugmentation with consortium-N							
k (day^{-1})	0.090	0.104	0.186	0.057	0.079	0.058	0.060
R^2	0.94	0.99	0.93	0.99	0.99	0.99	0.98
$t_{1/2}$ (days)	8	7	4	12	9	12	12
Solid-phase system							
(a) Natural attenuation							
k (day^{-1})	0.05	0.018	0.017	0.026	0.025	0.020	0.019
R^2	0.99	0.89	0.93	0.98	0.97	0.81	0.54
$t_{1/2}$ (days)	14	39	41	27	28	36	37
(b) Bioaugmentation with consortium-A							
k (day^{-1})	0.061	0.027	0.021	0.040	0.031	0.027	0.038
R^2	0.92	0.88	0.63	0.97	0.87	0.77	0.78
$t_{1/2}$ (days)	11	25	33	17	22	26	18
(c) Bioaugmentation with consortium-N							
k (day^{-1})	0.073	0.033	0.027	0.030	0.035	0.045	0.058
R^2	0.93	0.94	0.99	0.97	0.99	0.96	0.94
$t_{1/2}$ (days)	10	21	26	23	20	15	12

microcosms, the biodegradation rate constant was high in treatments that received consortium N. The highest biodegradation rate constant was observed for Ant (0.186 day^{-1}) followed by Ace (0.09 day^{-1}) and Pyr (0.079 day^{-1}) in slurry phase. In the solid phase, the maximum biodegradation rate constant was accounted in Ace (0.073 day^{-1}), BkF (0.058 day^{-1}), and BaA (0.045 day^{-1}).

4 Discussion

Microbial consortia have been used in several studies to successfully bioremediate PAH-contaminated soils. However, most studies used spiked soils and only the studies by Li et al. (2008), Mao et al. (2012), and Thavamani et al. (2012) as described in Table 2 employed long-term PAH-contaminated soils. Mao et al. (2012) found that a combination of bacteria isolated from real contaminated soils as consortium with autochthonous microbes caused a significant amount (20–36 %) of PAHs to be removed after 8 weeks in a model soil collected from an industrial area in China. Li et al. (2008) found that in bioremediation of a low PAH-contaminated soil for 4 weeks in solid vs. slurry microcosms, PAHs were significantly degraded (37–48 %) and maximum degradation was observed when the bacterial consortium was augmented in solid-phase microcosm compared to slurry-phase microcosm. However, the tested consortium enhanced 15–23 % of PAH degradation over natural attenuation. In the study by Thavamani et al. (2012), a bacterial consortium enriched from long-term PAHs and metal mixed contaminated soils with the HMW PAH degradation and metal tolerance as the significant traits, removed 38 % of total PAHs from a MGP soil over natural attenuation in 8 weeks.

Compared to previous studies, our analysis showed that after bioremediation for 15 weeks, 72–94 % of total PAHs removal was detected in the soil in solid and

Table 2 Bacterial consortium bioaugmentation of long-term PAHs contaminated soils in our and previous studies: an overview

Parameters	References				
Site	Mao et al. (2012) Industrial area	Li et al. (2008) Irrigation area	Thavamani et al. (2012) MGP site	This study MGP site	This study MGP site
Location	China	China	Australia	Australia	Australia
Initial PAHs (mg kg^{-1})	9.4	15.7	889	3960.7	3960.7
Consortium	*Mesorhizobium* sp., *Alcaligenes* sp., *Bacillus* sp.	NR	*Alcaligenes* sp., *Pseudomonas* sp., *Pandorea* sp., *Paenibacillus* sp.	*Pseudomonas* sp., *Stenotrophomonas* sp., *Agrobacterium* sp., *Trabulsiella* sp., *Cupriavidus* sp.	*Pseudomonas* sp., *Cupriavidus* sp., *Bacillus* sp.
Inoculation rate (%)	[a] 10 [b] 20	2	2	5	5
Type of microcosm	Solid	[1] Solid [2] Slurry	Slurry	[1] Solid [2] Slurry	[1] Solid [2] Slurry
Duration (weeks)	8	4	8	15	15
PAHs studied	Flu, Phe, Ant, Flt, Pyr, BaA, Chr, BbF, BkF, BaP	Ace, Acy, Nap, Ant, Flu, Phe, Chr, Flt, Pyr, BaA, BaP, BbF, BkF, DahA, IcdP	Ace, Acy, Nap, Ant, Flu, Phe, Chr, Flt, Pyr, BaA, BaP, BbF, BkF, DahA, IcdP	Ace, Phe, Ant, Flt, Pyr, BaA, BkF	Ace, Phe, Ant, Flt, Pyr, BaA, BkF
PAHs degradation (%)					
Natural attenuation	7.1	[1] 25.2 [2] 22.5	8.7	[1] 62.7 [2] 88	[1] 62.7 [2] 88
Bioaugmentation	[a] 20.2 [b] 35.8	[1] 47.7 [2] 37.1	46.6	[1] 72 [2] 92.6	[1] 73.4 [2] 93.7

NR not reported

slurry phases with the addition of 5 % consortium. Observed PAH losses were probably the result of both primary utilization and co-metabolism aided by autochthonous microbial populations (Mueller et al. 1990), since 63–88 % of total PAH removal was observed in the untreated soil (natural attenuation). Adding inoculum contributed to 6–11 % of total PAHs removed from the tested soil which is less than in previous reports. Co-metabolism might have played a major role in bioremediation, where HMW PAHs might be co-metabolized by the enzymes induced through LMW PAHs or a few specific metabolites, for example, salicylate during biodegradation (Chen and Aitken 1999; Wang et al. 2010). Other reasons for significantly higher PAHs degradation in the current study over previous ones could be higher inoculation rate (5 %), longer incubation time and presence of not more than seven different PAHs compounds in the tested soil.

As Nam et al. (2001) pointed out, the rate and extent of LMW PAH biodegradation occurs more rapidly and extensively than that of HMW PAHs, since the microbes generally pefer to utilize simple carbon source first as the source of energy and leave the complex substrates for later use (Phale et al. 2007). However, degradation of HMW PAHs could be greater under some circumstances. For instance, Li et al. (2008) found significantly higher degradation of ≥5-ring PAHs compared to <5-ring PAHs. In our study, degradation of three- to four-ring PAHs was less than that of five-ring, BaA and BkF in solid-phase system. This could be perhaps due to the bacterial consortium being enriched from a soil containing a higher HMW/LMW ratio which subsequently would have resulted in better HMW PAH degradation capability. The more the initial concentration of individual PAHs, the higher was the percentage of PAH removal (as in the case of Pyr). This result was on par with previous findings (Alexander 2000; Li et al. 2008), which could be explained by the fact that the mass transfer rate of PAHs to microorganisms is influenced by the PAH concentration gradients that consequently lower the toxicity for aged contaminants. Notably, during the whole period of incubation, sequential utilization of PAHs which generally occurs in a multi-substrate system due to competitive inhibition among the substrates was not apparent in both systems (Stringfellow and Aitken 1995; Desai et al. 2008).

In this study, the degradation of PAHs in slurry was more than that in solid phase during both bioaugmentation and natural attenuation. These discrepancies could be due to limited availability of additives primarily oxygen, electron acceptors, soil/water ratios, and availability of contaminants to microorganisms in solid phase, which are substantially found at increased rates in a slurry system (Doick and Semple 2003; Nano et al. 2003; Collina et al. 2005). Since notable PAH degradation was also observed in natural attenuated soil, particularly for HMW PAHs involving BaA and BkF which are generally recalcitrant to microbial attack, we believe that the indigenous microbial community might possess the LMW and HMW PAH-degrading enzymes due to long-term adaptation. That was the reason why natural attenuation was almost on par with bioaugmentation in the selected MGP soil. Further, maximum degradation of PAHs by consortium N is attributed to the presence of biosurfactant-producing bacteria which enhanced the PAH degradation compared to consortium A that lack such strains.

We observed that the population of PAH degraders (monitored using MPN assay) and total microbes (measured as microbial activity in terms of FDA hydrolysis) significantly increased the microbial population with addition of the bacterial consortium especially during the first 5 weeks and then declined by the end of the experiment. This could be directly related to the biodegradation efficiency, i.e., decline in microbial population with subsequent decline in the bioavailable PAH fractions. It is important to note that if the augmented microbes would have survived, then PAH biodegradation would have been more rapid than observed in the current study. This finding could mean that the microorganisms released into the new environment might have had difficulty in establishing themselves in the new ecological niche as stated by Ho and Banks (2006). Indigenous microbes with high metabolic activity could have suppressed the population of the introduced strains. This phenomenon states that although adding foreign organisms can affect the soil microbial number and structure, it cannot stay stable for long because the soil's physical, chemical, and/or biological complexity may cause a decline in the population of exogenous microbes (Mao et al. 2012). Therefore, it is necessary to study the performance of the developed microbial consortium using only a few other real contaminated soils with less dominant indigenous microbial community in order to confirm the potential of our consortia to bioremediate PAHs in field soils.

Table 3 PAHs half-life ($t_{1/2}$) values in our and previous studies: an overview

Parameters	References					
	Thiele-Bruhn and Brümmer (2005)		Wang et al. (2007)		This study	
General details						
Soil	Industrial site, Germany		Steel-manufacturing factory site, China		MGP site, Australia	
PAH degraders	Indigenous		Indigenous+bacterial consortium		Indigenous+bacterial consortium	
Condition	Solid-phase system		Slurry-phase systems		Solid- and slurry-phase systems	
Incubation period	168 weeks		26 weeks		15 weeks	
PAH degradation kinetics						
	Initial conc.	$t_{1/2}$	Initial conc.	$t_{1/2}$	Initial conc.	$t_{1/2}$
Ace	208	6–918	NR	NR	631	8–14
Phe	81	1.3–255	0.4	4.6	449	7–39
Ant	199	2.1–280	0.1	8.3	117	4–41
Flt	155	8.9–432	1.2	5.0	454	12–27
Pyr	98	25–1840	1.1	6.3	2252	9–28
BaA	28	40–2450	0.7	34	7	12–36
BkF	208	98–2220	0.4	201.9	56	12–37

Initial concentration is given in mg kg^{-1}, and $t_{1/2}$ values are given in days

NR not reported

The half-life values observed in the current study agree with the findings of Thiele-Bruhn and Brümmer (2005) and Wang et al. (2010) who found the highest residues to be those of BaA and BkF during the biodegradation of PAHs mix in long-term contaminated soils (Table 3). Our study was also in line with the results noted by Bossert and Bartha (1986) and Wang et al. (2010) who witnessed that Phe was more persistent than Ant. Relatively slow degradation of Flt could be explained in relation to its molecular structure (presence of nonaromatic ring structure), which resulted in slower degradation than in compounds with purely benzoid structures of similar molecular weight (Wang et al. 2010). To the best of our knowledge, the current study is the first report of the biodegradation kinetics of PAH mixtures during bioaugmentation and/or natural attenuation in long-term mixed contaminated soils such as MGP soil. Most half-lives observed in slurry-phase microcosms were much lower than solid-phase microcosms, which could be attributed to the improved mass transfer and bioavailability of PAHs in slurries as aforementioned. This indicates that bioreactor (bioslurries) could optimize microenvironments for microbes to ensure they maintain high levels of activity ex-situ. Thus, the microbes exhibited their utmost potential to degrade pollutants in aged soils.

5 Conclusions

Our study shows that microbes capable of hydrocarbon degradation are widespread in contaminated soils and they could play an important role in the natural dissipation of PAHs from the environment under feasible conditions, i.e., provided the soil is pulverizing, moistened, and aerated. It can be expected that PAHs in such soils can be bioremediated without further bioaugmentation and/or biostimulation. Furthermore, it is beneficial to conduct a careful investigation of a contaminated soil to select the most suitable bioremediation strategy since not every soil like that used in our study requires an intense remediation process. This is due to the existence of effective and efficient native pollutant metabolizers, where natural attenuation is more than enough to achieve successful bioremediation. The developed bacterial consortia may be promising candidates for bioremediation of other PAH-contaminated field soils.

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